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Quantification of surfactant protein D (SPD) in human serum by liquid chromatography-mass spectrometry (LC-MS)

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ABSTRACT

Quantification of intact proteins in complex biological matrices by liquid chromatography-mass spectrometry (LC-MS) is a promising analytical strategy but is technically challenging, notably for concentrations at or below the ng/mL level. Therefore, MS-based protein quantification is mostly based on measuring protein-specific peptides, so-called 'surrogate peptides', that are released through proteolysis. While quantitative protein bioanalysis based on peptide LC-MS is much more sensitive, not every peptide is suitable in this respect. For example, some peptides are too small to be unique for a protein while others are too large to be measured with sufficient sensitivity, so careful selection of appropriate peptides is essential. Here we present a validated LC-MS method for quantification of surfactant protein D (SPD) at clinically relevant levels between 5 and 500 ng/mL using 50 µL of serum. This method targets two SPD-specific peptides in the C-type lectin, ligand binding domain of the SPD protein. One of these peptides contains a methionine residue which would typically be avoided because of its unstable nature. Some quantitative methods do target methionine-containing peptides, and corresponding workflows feature an oxidation step at the peptide level using hydrogen peroxide (H_2O_2) to convert all methionine residues to more stable methionine sulfoxides. For our method, such a procedure was associated with peptide loss, hence we developed an oxidation procedure at the protein level using H_2O_2 to oxidize methionine residues and the enzyme catalase to quench excess H_2O_2. This procedure may be applicable to other quantitative methods based on a surrogate peptide-based approach and may potentially also be useful for MS-based workflows targeting intact proteins.

1. Introduction

Over the past decades, mass spectrometry (MS) has emerged as a robust and versatile analytical platform in the study of proteins [1,2]. MS-based methods can, for example, provide insights into the structure and composition of these complex biomolecules or can be used to quantify proteins in a wide range of sample matrices [3]. The latter application holds considerable potential for addressing key questions in fundamental research, yet this application is interesting from a clinical diagnostic point of view as well [4]. However, MS-based protein quantification is also associated with several challenges and often requires method developers to make an important conceptual decision upfront, namely whether a protein will be studied in its intact form or by means of its peptides, which are typically released through proteolysis [5].

Quantitative methods that target protein-specific peptides, so-called 'surrogate peptides', represent the vast majority of all targeted proteomics methods, and peptides are furthermore often measured in

Abbreviations: ABC, ammonium bicarbonate; ACN, acetonitrile; AF, allele frequency; BSA, bovine serum albumin; CID, collision-induced dissociation; COPD, chronic obstructive pulmonary disease; CV, coefficient of variation; DDA, data-dependent acquisition; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; EMA, European Medicines Agency; ESI, electrospray ionization; FA, formic acid; FDA, Food and Drug Administration; H_2O_2, hydrogen peroxide; IAM, iodoacetamide; IS, internal standard; IVD, *in vitro* diagnostic; LC, liquid chromatography; LLOQ, lower limit of quantification; MS, mass spectrometry; nsSNP, nonsynonymous single nucleotide polymorphism; PBS, phosphate buffered saline; PTM, post-translational modification; QC, quality control; rh-SPD, recombinant human surfactant protein D; SIL, stable isotope labelled; SPD, surfactant protein D; TCEP, tris(2-carboxyethyl)phosphine; TFA, trifluoroacetic acid

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discovery-based proteomics experiments [6–9]. Not every peptide, however, can be used to identify or quantify a protein of interest. For example, very small peptides often do not have a unique sequence and are thus susceptible to interferences, while very large peptides may suffer from unfavorable analytical behavior (*e.g.* hydrophobicity-related peptide adsorption or peak tailing upon reversed-phase liquid chromatography (LC)) [10,11]. Peptide selection for quantitative workflows accordingly is a crucial and oftentimes tedious task, and several critical criteria need to be taken into account [12]. While there are no consensus criteria for peptide selection, most recommendations in literature give similar instructions with regard to peptide length, peptide uniqueness, the absence of post-translational modification (PTM) sites, proteolytic digestion efficiency, and physicochemical properties in relation to the MS detection, notably electrospray ionization (ESI) efficiency and fragmentation upon collision-induced dissociation (CID) [10–14]. Some publications furthermore recommend avoiding peptides that feature reactive amino acid residues such as cysteines (oxidation), methionines (oxidation), N-terminal glutamines (cyclization), and asparagines or glutamines when followed by a glycine (deamidation) [13,14]. Remarkably, only few publications take genetic variation into account and recommend avoiding peptides that are prone to modification due to highly prevalent nonsynonymous single nucleotide polymorphisms (nsSNP) [11–13]. The above-mentioned set of criteria make it clear that peptide selection can be a challenging task, and taking all of these criteria into account greatly reduces the options for studying a protein by means of its proteolytic peptides. Therefore, strategies are frequently being used to render unfavorable peptides suitable for LC-MS by modifying their properties through chemical reactions (*e.g.* cysteine carbamidomethylation using iodoacetamide, full methionine oxidation using hydrogen peroxide) and thereby increasing the options for reliably quantifying a given protein of interest [15–17].

Surfactant protein D (SPD) is one of the four surfactant proteins present in human pulmonary surfactant, a complex mixture of lipids and proteins that covers the respiratory epithelium and maintains a low surface tension thereby preventing the lungs from collapsing [18]. Moreover, SPD is an important component of the innate immune response to various pathogens including bacteria, fungi, and viruses [19]. This protein has been proposed to play a key role in several pulmonary disorders and is considered to be a promising biomarker for asthma, chronic obstructive pulmonary disease (COPD), and interstitial lung disease [20–24]. Its current status as so-called 'biomarker candidate' is based on measurements with a small number of commercial enzyme-linked immunosorbent assay (ELISA) kits, and corresponding SPD levels, as reported in literature, were found to exhibit considerable variation [25]. The development of SPD as biomarker would thus benefit from alternative methodologies that generate defined chemical information rather than measuring SPD indirectly, as is the case for immunoassays.

In this study, we developed an LC-MS method for quantification of SPD in human serum at clinically relevant ng/mL levels and validated this method following requirements as stipulated in the European Medicines Agency (EMA) and United States Food and Drug Administration (FDA) guidelines [26,27]. SPD quantification was based on a surrogate peptide approach by targeting two tryptic peptides in the C-type lectin, ligand binding domain of SPD. One of these peptides contains a methionine residue, which could be readily oxidized to the sulfoxide by treating samples with H_2O_2 after tryptic digestion. However, this procedure gave rise to peptide loss, which was incompatible with a validated quantitative bioanalytical method. To overcome this limitation, we developed an oxidation procedure at the protein level using H_2O_2 which is followed by an incubation step with catalase to quench excess H_2O_2 to arrive at a validated assay for SPD quantification.

2. Experimental

2.1. Chemicals and materials

Recombinant human SPD (rh-SPD; Cat. No. 1920-SP; UniProtKB ID 'P35247'; Ala21-Phe375 with a Glu22Gly substitution) and monoclonal anti-SPD antibody (Cat. No. MAB1920; clone 292201) were purchased from R&D Systems (Abingdon, U.K.). Stable-isotope-labelled (SIL) SPD peptides carrying ^{13}C- and ^{15}N-labelled lysine residues (*i.e.* NEAAFLSMTDSK and SAAENAALQQLVVAK) were synthesized by Thermo Scientific's Peptide Synthesis Services and were obtained as crude peptides (≥99% isotopic purity, peptide purity unspecified (unpurified), not quantified) via Life Technologies Europe B.V. (Bleiswijk, The Netherlands). Acetonitrile (ACN; LC-MS grade) was obtained from Biosolve (Valkenswaard, The Netherlands) and sequencing grade modified trypsin was purchased from Promega (Madison, WI, U.S.A.). Nunc-Immuno™ MicroWell™ 96 wells plates with MaxiSorp™ coating (Cat. No. M9410), bovine serum albumin (BSA; Cat. No. A7638), catalase (from bovine liver; 2–5 kU/mg; Cat. No. C9322), hydrogen peroxide (H_2O_2; 30% (w/w) in water; Cat. No. 216763), and phosphate buffered saline (PBS; 10 × ; Cat. No. D1408) as well as all other chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

2.2. Serum samples

Pooled serum from healthy human subjects (obtained from Seralab, West Sussex, U.K.) was used for preparation of the quality control (QC) samples. The serum was either used directly as QC-low sample or was fortified with rh-SPD at two levels to obtain the QC-medium and QC-high samples. For recovery and spike-recovery experiments, six individual sources of serum from healthy human subjects were used as well as a lipemic serum sample (triglyceride content > 150 mg/dL) and a hemolytic sample that was prepared by adding freeze-thawed whole blood (2%) to serum from a healthy human subject (all from Seralab).

2.3. Calibrants and internal standard

Lyophilized SPD was dissolved in Milli-Q water to obtain a 500 µg/mL solution (based on the quantity as declared by the supplier, which was confirmed by quantitative amino acid analysis), which was diluted to 100 µg/mL with 1 × PBS, pH 7.4 (PBS Buffer). The resulting solution was diluted to 10 µg/mL with 1% BSA in PBS Buffer (Surrogate Matrix), and calibration samples were prepared at 5, 10, 20, 50, 80, 100, 200, 400, and 500 ng/mL in Surrogate Matrix. The internal standard (IS) stock solution was prepared by diluting the two SIL-peptides (supplied as crude solutions in 0.1% trifluoroacetic acid (TFA) in 50% ACN) with 1% dimethyl sulfoxide (DMSO) in water to a 100 × stock solution which, upon dilution in Digestion Buffer (see below), matched the middle of the calibration curve (approx. 50 ng/mL).

2.4. Antibody-based SPD capture and in-well digestion

The presented method is analogous to a previously published method featuring a sequential microtiter plate-based immunoaffinity enrichment and in-well digestion protocol [28], with the exception of a methionine oxidation step. Briefly, microplate wells were coated overnight with 0.5 µg of anti-SPD antibody in PBS Buffer, washed with Wash Buffer (0.05% Tween-20 in PBS Buffer), blocked for 60 min with Blocking Buffer (1% BSA in PBS Buffer), and washed again with Wash Buffer. Next, 100 µL of Sample Solution (1:1 mixture of serum or calibrant and Surrogate Matrix) was added to the wells, and the plates were incubated for 180 min. After washing the wells with Wash Buffer, 100 µL of Digestion Buffer (1 × IS stock solution in 100 mM ammonium

bicarbonate (ABC) containing 10 mM TCEP) was added to the wells, plates were sealed, and disulfide bonds were reduced for 30 min at 60 °C. After cooling the plates to room temperature, thiols were alkylated in 20 mM iodoacetamide (IAM) for 30 min in the dark after which non-reacted IAM was quenched with a 0.5 molar equivalent of DTT for 5 min. Methionines were subsequently oxidized in 1% hydrogen peroxide (H_2O_2; 5 µL 23% H_2O_2 in water) for 20 min (15 min on a plate shaker (900 RPM) and 5 min in a plate centrifuge) at room temperature, and the excess of H_2O_2 was afterwards quenched with 0.5 µg catalase (1–2.5 U; 5 µL 0.1 µg/µL catalase in ABC) by incubation for 55 min on a plate shaker (900 RPM) and 5 min in a plate centrifuge at room temperature. Lastly, proteins were digested with 200 ng of trypsin overnight at 37 °C, and the digests were acidified through addition of formic acid (FA) after which they were stored in the autosampler of the LC-MS system at 10 °C until analysis.

2.5. Targeted analysis by LC-MS

Analyses were performed with a Waters Ionkey/MS system using an ACQUITY M-Class UPLC and a XEVO TQ-S mass spectrometer (Milford, MA, U.S.A.) operating in the multiple reaction monitoring (MRM) mode. 15 µL of sample was loaded onto a C18-bonded trap column (Dionex, Cat. No. 160454) for 2.5 min at 20 µL/min with 3% mobile phase B (0.1% FA in ACN) in mobile phase A (0.1% FA in water). Trapped peptides were subsequently separated on a C18-bonded iKey LC column (Waters, Cat. No. 186007261) kept at 40 °C with an 8-min linear gradient from 3 to 27% B at 3 µL/min, after which the column was cleaned (1 min at 60% B and 5 min at 95% B) and equilibrated (3 min at 3% B). MRM transitions and settings for SAAENAALQQLVVAK and NEAAFLSMTDSK are presented in Table A.1, and specific MS operation conditions have been described in detail elsewhere [28].

2.6. Method validation

The method was validated following requirements as stipulated in the most recent versions of the EMA and FDA guidelines on bioanalytical method validation [26,27]. Specifically, the following criteria were addressed: calibration curve, accuracy & precision, recovery, selectivity (*e.g.* spike recovery), and stability (*e.g.* 27 days benchtop, 5 × freeze-thaw, and 20 days autosampler (10 °C)). Corresponding experimental procedures have previously been described in detail [28].

2.7. Method comparison and testing

For method comparison, 32 serum samples were analyzed from a cross-sectional study (NCT00807469) within the University Medical Center Groningen (UMCG) [29]. This study was ethically approved by the UMCG's review board (METc 2008/136) and adheres to the Declaration of Helsinki. Blood samples were collected as described previously [30]. In all samples, SPD was quantified using the commonly-used Surfactant Protein D Human ELISA from BioVendor (Cat. No. RD194059101) as well as the novel LC-MS method. The LC-MS method was furthermore tested by measuring 179 samples from the above-mentioned clinical study, of which 32 had already been analyzed for method comparison purposes. For this work, we also performed incurred sample reanalysis on 20 serum samples, which corresponds to 11% of the total number of samples, in agreement with EMA and FDA recommendations [26,27].

3. Results and discussion

3.1. Method development

We aimed to develop a surrogate peptide-based LC-MS method for quantification of SPD in human serum at clinically relevant ng/mL levels which employs an external calibration (*i.e.* using calibration samples) strategy as well as stable isotope labelled (SIL-)peptides as internal standards, given that a SIL-version of the full-length SPD protein was not available. In addition, we aimed to include, at least, two peptides which may prove valuable in case interferences are encountered when applying the method to clinical studies, which feature samples that are not as 'predictable' as the (commercial) sera that are typically used for method development and method validation purposes. Inspired by the multitude of recommendations for peptide selection [10–14], we furthermore aimed to select peptides that are of favorable length (7–20 amino acids), lack unfavorable amino acid residues (carrying post-translational modifications) or sequence motifs (*e.g.* deamidation-prone NG and QG sequence motifs, cyclization-prone N-terminal glutamines), lack highly prevalent nsSNPs, and are unique for the human SPD protein (Ensembl gene ID 'ENSG00000133661'). Only few peptides of the 35 kDa SPD protein meet these criteria when considering trypsin as well as six alternative proteases (see Figs. A.1 and A.2) [31]. Five tryptic peptides were eventually tested, of which two, NEAAFLSMTDSK (position 308–319) and SAAENAALQQLVVAK (position 293–307), were reliably detected in serum. Both of these peptides are located in the C-type lectin, ligand binding domain of SPD. The NEAAFLSMTDSK peptide is furthermore expected to be absent in approximately 1% of the population due to a nsSNP (*i.e.* rs4469829) giving rise to a Glu309Lys substitution (based on data from the ExAC Browser [32]). In subjects carrying this nsSNP, an N-truncated version of the NEAAFLSMTDSK peptide can be expected which lacks the first two amino acids. As this nsSNP occurs in only 1% of the population, we did not focus on this mutation, but the sequence of this truncated peptide is still unique for the SPD protein, hence it is, in principal, possible to include it as surrogate peptide.

The NEAAFLSMTDSK peptide contains an oxidation-prone methionine residue which does not necessarily affect its suitability as quantifier peptide. However, we observed peptide loss when applying a published approach [17] to oxidize methionine residues using hydrogen peroxide (H_2O_2) at the peptide level, which may be due to reactive oxygen species that are formed following the addition of H_2O_2. We therefore adopted an H_2O_2 oxidation step at the protein level directly followed by H_2O_2 quenching with catalase, an enzyme that is present in aerobic organisms and that catalyzes the dismutation of two H_2O_2 molecules into oxygen and water. SPD denaturation prior to the oxidation step was needed in order to fully oxidize Met^{315} at the protein level (see Fig. A.3). In addition, we needed to optimize the duration of the oxidation step as well the percentage of H_2O_2 that was used (see Fig. A.4). We furthermore increased the rotation speed and introduced intermediate centrifugation steps for both the oxidation and the H_2O_2 quenching procedures in order to remove oxygen bubbles from the sample solution (see the Experimental section for the final procedure). Lastly, we verified the relevance of the H_2O_2 quenching step (see Fig. A.5) prior to validating the method according to regulatory guidelines.

3.2. Method validation

Table 1 summarizes the validation results. A full overview of all validation results is provided in Tables A.2 to A.21. The LC-MS method for SPD quantification based on a surrogate peptide approach was validated following requirements as stipulated in the most recent versions of the EMA and FDA guidelines on bioanalytical method validation [26,27]. This method employs an adsorptive microtiter plate-based immunoaffinity enrichment procedure for which we demonstrated that using 0.5 µg of antibody per sample allowed for the enrichment of SPD from 50 µL of human serum (see antibody titration results in Fig. A.6) with good precision (< 10%; see Tables A.2 and A.3). Accurate SPD quantification was furthermore shown for a 1/x-weighted linear calibration model using 9 non-zero calibrants with recombinant human SPD (rh-SPD) levels ranging from 5 to 500 ng/mL (see Tables A.4 and A.5 as well as Fig. A.7 which features typical examples of chromatograms for the 5 ng/mL calibration samples). For preparation of the

Table 1
Summary of validation data [a].

		NEAAFLSMTDSK		SAAENAALQQLVVAK	
		max. CV	max. bias[b]	max. CV	max. bias[b]
calibration curve (5 runs, 9 non-zero calibrants)		7%	4%	7%	4%
accuracy & precision (3 runs, in 6-fold)	LLOQ	20%	19%	14%	−12%
	QC-low	8%	13%	6%	10%
	QC-medium	11%	2%	14%	(−)3%
	QC-high	5%	6%	4%	3%
autosampler stability 10 °C (20 days, in 3-fold)		6%	−12%	9%	−13%
bench-top stability (27 days, in 3-fold)		9%	−12%	4%	−13%
freeze-thaw stability −20 °C (5 cycles, in 3-fold)		5%	14%	7%	9%

	NEAAFLSMTDSK			*SAAENAALQQLVVAK*		
	recovery	**CV**	**bias**	**recovery**	**CV**	**bias**
recovery (6 different serum samples)	76%	7%	–	78%	9%	–
spike recovery (6 different serum samples)	95%	14%	−5%	99%	15%	−1%
lipemic sample spike recovery (in 2-fold)	71%	–	−29%	73%	–	−27%
hemolytic sample spike recovery (in 2-fold)	98%	–	−2%	96%	–	−4%

[a] An extensive summary of the validation results is presented in Tables A.2 to A.21.
[b] The average value of measured concentrations during the accuracy and precision experiments was used as nominal concentration for the accuracy and precision experiments (QC-low, QC-medium, and QC-high) and the stability assessments.

calibrants, 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) was used as surrogate matrix. This matrix was found to adequately mimic human serum with regard to the enrichment of rh-SPD and the subsequent sample preparation procedures (see the responses and overlapping 95% confidence intervals for the slopes of 'Human Serum' and '1% Bovine Serum Albumin' in Fig. A.8).

Evaluation of accuracy and precision revealed acceptable biases and CVs (within ± 20%) for the lower limit of quantification sample (LLOQ; see Tables A.6 and A.7) as well as acceptable biases and CVs (within ± 15%) for the QC-low, QC-medium, and QC-high samples (see Tables A.8 to A.13). Acceptable biases and CVs (within ± 15%) were also observed for all stability assessments (see Tables A.14 to A.19) which indicates that SPD is a rather stable protein, at least based on the site that is recognized by the anti-SPD antibody and the two surrogate peptides that are used for SPD quantification.

Selectivity was assessed on the basis of spike recovery experiments which were carried out on six different sources of human serum, a lipemic sample, and a hemolytic sample. These experiments resulted in recoveries close to 100% except for the lipemic sample which featured a recovery of 71–73% (see Tables A.20 and A.21). Interpretation of these results should be done prudently as corresponding spiking procedures were carried out using rh-SPD. This surrogate protein calibrator may not fully reflect the heterogeneity of circulating SPD forms (so-called proteoforms [33] or protein species [34]) thus potentially leading to variance in immunoaffinity enrichment and digestion efficiencies. In addition, it should be taken into account that circulating SPD mainly originates from the lungs and leaks out from lung compartments into the circulation [35,36]. The corresponding uptake (mechanism and efficiency) of endogenous SPD in blood may be different from the uptake of recombinant SPD in serum during the spike recovery experiments, which represents another potential explanation for deviating spike recovery results that needs to be addressed in further studies.

3.3. Method comparison

The LC-MS method for SPD quantification was compared with an ELISA, which is registered in the European Union for *in vitro* diagnostic (IVD) use, on the basis of 32 human serum samples [29]. This comparison revealed good correlation between the methods (R^2 = 0.9) but indicated also that the LC-MS method yields approximately 1.5 times higher SPD levels than the ELISA (see Fig. 1). As most of the samples included in this comparison are pairs of samples from different time points belonging to the same patients, we were also able to compare both methods on the basis of the ratio of SPD levels for these sample pairs (N = 14). Again, correlation between the methods was good (R^2 = 0.9) (see Fig. A.9). Since both methods were referenced against the same SPD protein standard and because all measured SPD levels were within the linear range of the ELISA's calibration curve, it is likely that the observed 1.5-fold difference is related to the different detection principles of the assays, for example, due to varying specificities and/or binding efficiencies of the antibody that is used for the LC-MS method and those that are used by the ELISA (binding sites of the antibodies are unknown). In addition, it should be taken into account that the ELISA measures intact SPD while the LC-MS method includes a proteolytic digestion step and analyzes SPD by means of tryptic peptides, which represent two fundamentally different approaches to quantify a protein of interest.

A comparison between the detection principles of the assays is not straightforward, or feasible, as the ELISA relies on a secondary detection antibody to bind SPD proteins in order to generate a signal, while the LC-MS method detects and quantifies SPD based on protein-specific surrogate peptides. We were, however, able to make a (preliminary) comparison between the capturing steps of both methods by analyzing 6 human serum samples and two calibration standards with the validated LC-MS method as well as with a modified version of this method in which we used the BioVendor ELISA plate for immunoaffinity enrichment and in-well digestion prior to LC-MS. This experiment revealed a very good correlation between both approaches ($R^2 \geq 0.98$; see Fig. A.10) suggesting that both methods capture a comparable fraction of SPD proteoforms. Nevertheless, the evidence to support this conclusion is circumstantial, and the exact nature of the observed bias between both methods has yet to be determined.

The concept of providing a mass spectrometric readout for a commercial ELISA kit was furthermore adopted for a data-dependent acquisition (DDA) shotgun proteomics experiment (see Method A.1). Here we attempted to identify SPD and potentially also detect SPD peptides carrying posttranslational modifications, such as hydroxylated proline or lysine residues in the N-terminal, collagen-like domain of the protein. We furthermore searched for SPD peptide sequences that feature substituted amino acid residues due to one of the five most prevalent nsSNPs that are listed in the ExAC Browser [32], namely rs2243639 (Thr180Ala, AF = 0.66), rs721917 (Met31Thr, AF = 0.47), rs3088308 (Ser290Thr, AF = 0.09), rs17878336 (Leu123Val, AF = 0.03), and rs4469829 (Glu309Lys, AF = 0.01). Based on four human serum

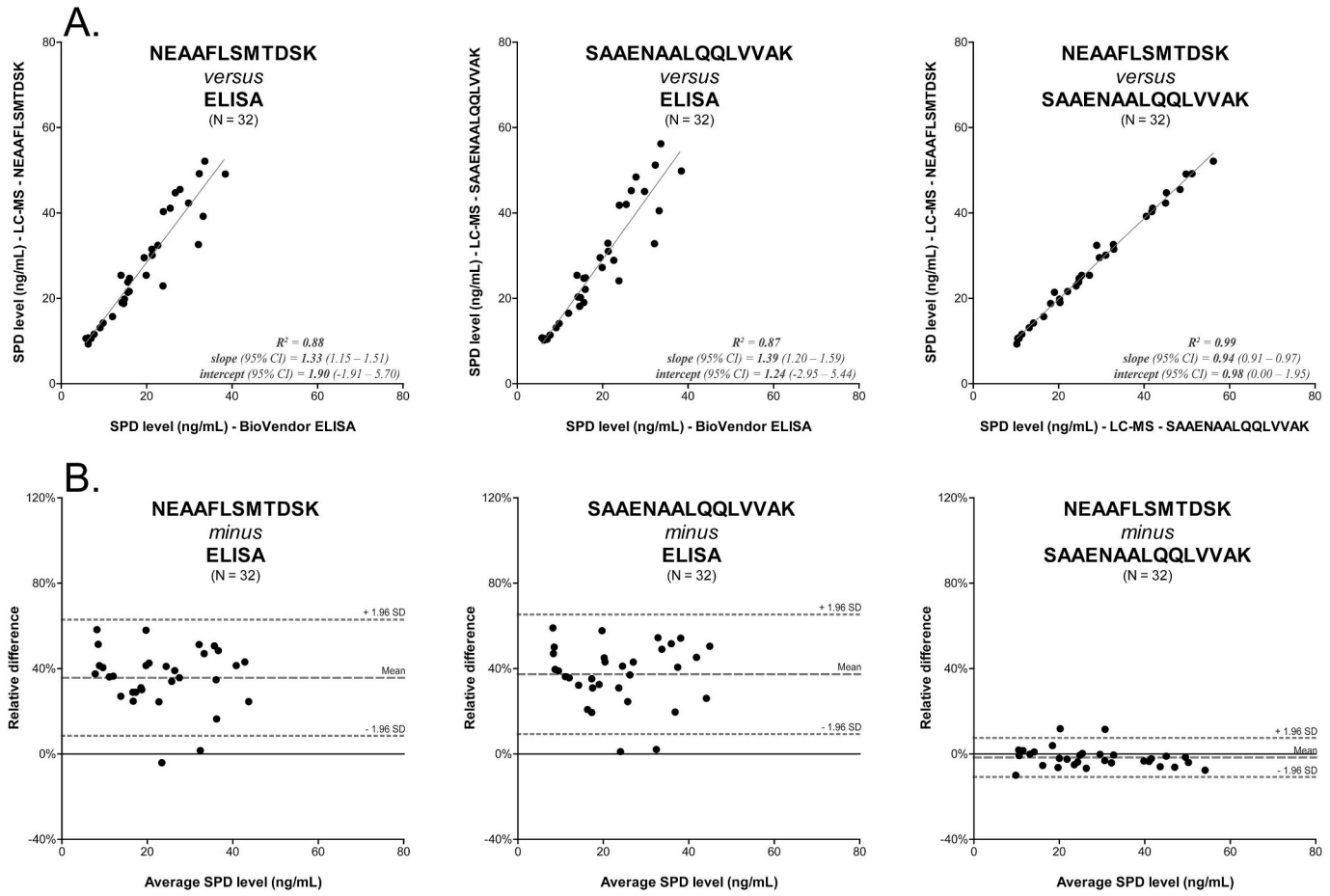

Fig. 1. Comparisons between the quantitative LC-MS SPD method (both the NEAAFLSMTDSK and the SAAENAALQQLVVAK peptides) and the BioVendor Surfactant Protein D Human ELISA (Cat. No. RD194059101) using (**A**) linear regression and (**B**) Bland-Altman plots.

samples and by using two different proteases, we identified SPD in all samples, detected hydroxylated proline residues in two samples, and obtained mass spectrometric evidence for the presence of two nsSNPs (*i.e.* rs721917, rs3088308) in one sample (see Figs. A.11 and A.12).

These experiments and corresponding findings highlight the scope of this method and other surrogate peptide-based LC-MS methods. This direction is instigated by the remarkable (and still improving) speed and sensitivity of modern mass spectrometers. These instruments can, on the one hand, be used to provide a protein quantity and, on the other hand, may yield structural information of a protein target of interest, which represents a distinct characteristic of MS-based assays as compared to conventional immunoassays.

3.4. Method testing

The LC-MS method was applied to 179 clinical samples [29] (analysis and interpretation of the clinical data will be reported in future publications) in order to study the method's performance further. These analyses showed that the method features excellent inter-peptide agreement (see Fig. 2 and Fig. A.13) thereby supporting the initial peptide comparison data that were obtained during the method comparison experiment (see the plots on the right in Fig. 1A and B). We furthermore performed incurred sample reanalysis according to EMA and FDA recommendations [26,27] to verify the reliability of the reported SPD levels. These experiments revealed that 80% and 85% of the repeat analyses for SAAENAALQQLVVAK and NEAAFLSMTDSK, respectively, yielded SPD levels with a relative difference within ± 20%

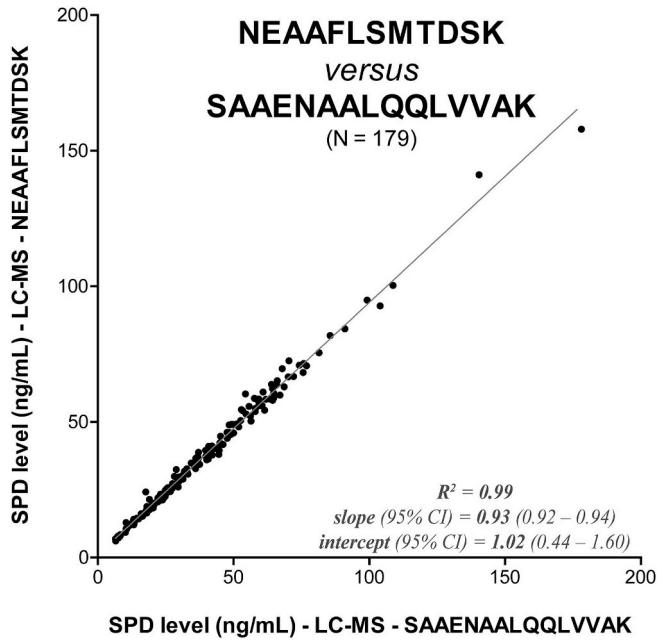

Fig. 2. Comparisons between SPD levels calculated on the basis of the tryptic SPD peptides NEAAFLSMTDSK and SAAENAALQQLVVAK using linear regression. The corresponding Bland-Altman plot is shown as Fig. A.13.

(see Fig. A.14). These percentages are well above the 67%, which is specified as cut-off value for chromatographic assays in the EMA and FDA guidelines [26,27] and thus underline the adequate performance of the presented SPD method.

4. Conclusion

We describe a surrogate peptide-based LC-MS method for quantification of SPD in human serum which complies with current international guidelines on bioanalytical method validation. The method shows good correlation and agreement with an IVD-grade ELISA kit, however, the LC-MS method reports approximately 1.5 times higher SPD levels compared to the ELISA. The exact reasons for this difference are still unclear, yet the difference may be related to the fact that the ELISA measures intact SPD, while the LC-MS method analyzes the SPD protein by means of two tryptic peptides. It could, for example, be possible that the secondary, detection antibody of the ELISA is hampered by interactions between SPD and (some of) its protein binding partners, whereas such interactions are not expected to interfere with the LC-MS method due to inclusion of a trypsin digestion step.

A distinctive feature of the LC-MS method is the incorporation of an H_2O_2-based methionine oxidation step at the protein level followed by incubation with the enzyme catalase to quench excess H_2O_2. This combination may provide a useful alternative to conventional H_2O_2-based methionine oxidation procedures at the peptide level, in case such procedures give rise to peptide stability issues. In addition, this procedure may potentially be valuable for MS-based workflows targeting intact proteins. However, it must be kept in mind that protein size and three-dimensional structure need to be considered in light of the effectiveness of oxidation procedures at the protein level.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2019.05.028.

Appendix B. Supplementary Data

Data have been deposited to the ProteomeXchange (DDA) and PASSEL (MRM) repositories under accession codes ‘PXD013415’ and ‘PASS01363’, respectively.

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